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Germline mutations in *BRCA1* account for about 45% of all hereditary cases of breast cancer. However, the role of BRCA1 in mammary tumorigenesis is not established. My proposal outlines two approaches to extend our knowledge of BRCA1 function in normal development and malignant transformation. First, I used the *cre-lox* system to generate a mouse model in which loss of BRCA1 is limited to mammary epithelium. Four different mouse lines expressing the Cre recombinase in mammary epithelium were generated, and we are currently working towards generating a *lox-Brca1-lox* mouse. Secondly, we examined synergistic effects between BRCA1 and p53 in tumorigenesis. We found that survival of both $p53^{-/-}$ and $p53^{+/-}$ mice is not altered by BRCA1-deficiency. This suggests that BRCA1 does not play a role in the formation of most tumors. Similarly, the survival of the $p53^{+/-}$ mice after exposure to gamma-irradiation was unaltered by BRCA1-deficiency. However, while none of the irradiated $p53^{+/-}$ mice developed mammary tumors, five tumors isolated from $Brca1^{+/-}$ mice were of mammary epithelial origin and of these three had lost expression of the wild-type *Brca1* gene. These results are suggestive of a role for BRCA1 in mammary tumor formation after exposure to specific DNA-damaging agents.

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INTRODUCTION

Breast cancer is a common and important disease affecting women. It is estimated that one in eight American women who reach age 95 will develop breast cancer. An inherited component to breast cancer has long been suspected. It is now estimated that genetic factors contribute to about 5% of all cases of breast cancer and approximately 25% of cases diagnosed before age 30 (1). Mutation of one gene, *BRCA1*, is thought to account for approximately 45% of families with significantly high breast cancer incidence and at least 80% of families with increased incidence of both early-onset breast cancer and ovarian cancer (2-4). *BRCA1* appears to encode a tumor suppressor, a protein that acts as a negative regulator of tumor growth.

To understand how the inheritance of a mutant allele of *BRCA1* results in a predisposition to breast and ovarian cancer, a mouse model was generated in our laboratory by introducing a mutation in the murine homologue to *BRCA1*(5). The generation of these mice would also allow us to examine the role of BRCA1 in normal tissues. The *Brca1*$^{+/-}$ mice were found to be fertile and developmentally normal. Interestingly, unlike women with a single germline mutation in *BRCA1*, *Brca1*$^{+/-}$ mice did not have an increased risk of tumor formation of any type. Litters produced by mating these animals did not contain mice homozygous for the mutated allele (*Brca1*$^{-/-}$ mice). Analysis revealed that *Brca1*$^{-/-}$ embryos were resorbed between days 10 and 13 due to neural tube defects. While this discovery revealed an as yet unknown crucial function of BRCA1, it limited our ability to use this model to understand the function of BRCA1 in normal growth and development of mammary tissue.

In my proposal, I outlined two approaches to generate better mouse models to study the role of BRCA1 in mammary tumorigenesis. In the first technical aim, we proposed the generation of mice in which loss of BRCA1 is limited to the mammary gland epithelial cells. It was anticipated that this approach would circumvent the embryonic lethality associated with the loss of BRCA1 function and thereby allow us to examine the role of BRCA1 in the mammary epithelium. To accomplish this aim, a *cre-lox* targeting system was used to produce mammary-specific BRCA1-deficiency. The approach we used to achieve tissue-specific inactivation of the *Brca1* gene involves the use of a site-specific recombinase, the P1 Cre recombinase (6,7). This recombinase recognizes two short stretches of DNA (termed *lox* sequences) in a sequence-specific manner and catalyzes the deletion of DNA flanked by these recognition sites. This process can be made tissue-specific by placing the expression of the Cre recombinase under the control of a tissue-specific promoter, in our case the MMTV-LTR. Mice with an MMTV-*cre* transgene and mice containing *lox* sites flanking important exons of the *Brca1* gene are generated and bred to produce mice in which the Cre recombinase deletes *Brca1* only in the mammary epithelial cells.

In the second technical aim, I proposed to examine the possible cooperative effects between BRCA1 and another tumor suppressor, p53. By combining *Brca1* mutations with *p53* mutations, we hoped to initiate tumorigenesis in organs in which malignancy was absent in *Brca1*$^{+/-}$ mice. Mice homozygous for the *p53* mutated allele are viable and appear developmentally normal, but develop a variety of spontaneous tumors starting at ~6 weeks of age, while mice heterozygous for the mutated *p53* allele begin to develop tumors at 9 months of age (8,9). While neither the *p53*$^{+/-}$ nor *p53*$^{-/-}$ animals display an appreciable frequency of mammary adenocarcinomas, the rationale for this objective was based on the fact that high levels of p53 are observed in the breast during growth and remodeling, suggesting that it may be

involved in the apoptosis that accompanies this phase of mammary development (10). Examination of preneoplastic lesions in mice has also revealed the presence of *p53* mutations (11). Previous studies had demonstrated that, in some instances, animals carrying mutations in two different genes can display pathologies that are not observed when only one of these genes is mutated (12-14). Both p53 and BRCA1 are each thought to play a significant role in the progression of tumorigenesis in human mammary tissue. In this objective we were testing the hypothesis that BRCA1 and p53 display a synergistic interaction in promoting tumor formation, particularly in mammary epithelium.

I have over the past year made significant progress towards completing these aims. In the first technical aim, I generated eight MMTV-*cre* transgenic lines, and identified four lines which express the Cre recombinase in the mammary epithelium of pregnant females. I am currently using a transgenic mouse line which contains the β-galactosidase gene (*lacZ*) as marker for Cre expression to determine the variegation of Cre expression in the mammary epithelium in these four lines. In addition, after difficulty in generating a useful mouse line containing *lox* sites flanking exon 11 of *Brca1*, multiple approaches to generating this mouse line are currently underway.

Each of the proposed experiments in the second technical aim have been accomplished, and a manuscript containing our findings is currently in preparation. To determine whether mutations in *p53* would also modify the role of BRCA1 in mammary tumor formation in mice, I generated mice carrying mutations at both of these loci. A mutant *Brca1* allele was introduced into $p53^{+/-}$ and $p53^{-/-}$ mice and alterations in the rate of tumor formation and the tumor spectrum was monitored. Our studies have shown that the overall survival of both $p53^{-/-}$ and $p53^{+/-}$ mice is not altered by the presence of a mutant *Brca1* allele. Although no major changes in the tumor type were noted in either the $Brca1^{+/-}p53^{-/-}$ or $Brca1^{+/-}p53^{+/-}$ populations, four mammary tumors were seen in the $Brca1^{+/-}p53^{-/-}$ group while only one such tumor was seen among the control $p53^{-/-}$ group. It has been suggested that BRCA1 is involved in DNA repair particularly after exposure to gamma irradiation. We found that neither the overall survival rate nor the overall tumor spectrum of $p53^{+/-}$ mice after exposure to gamma irradiation was altered by the presence of a mutant *Brca1* allele. However while none of the 14 irradiated $p53^{+/-}$ mice developed mammary tumors, five of the 23 tumors isolated from the BRCA1-deficient mice were of mammary epithelial origin and of these three had lost expression of the wild type *Brca1* gene. Although the number of mammary tumors obtained was small, these results are suggestive of a role for BRCA1 in mammary tumor formation after exposure to specific DNA damaging agents.

In this review, I describe the experimental methods I have utilized to complete these technical aims and the results I have obtained from these studies. A discussion of the results and a conclusion summarizing my results will complete my review. I have subdivided each section into the two technical objectives I originally proposed: (1) Tissue-specific inactivation of BRCA1 and (2) Interactions with tumor suppressor genes.

EXPERIMENTAL METHODS

Technical Objective 1: Tissue-specific Inactivation of BRCA1

A. Generation of an MMTV-*cre* mouse. The MMTV-LTR drives expression in epithelial cells of both the mammary and salivary glands, as well as in lung, kidney, seminal vesicles and lymphoid cells. However, the highest levels of expression achieved with this

promoter are in mammary epithelium. The MMTV-LTR was obtained as the pMSG plasmid from Promega. Plasmids containing the *cre* gene were obtained from K. Parker and B. Sauer. Two different constructs containing the MMTV-LTR and a *cre* gene were made. Both constructs were transfected together with an *Hprt* gene into ES cells. Cells expressing the *Hprt* transgene were analyzed by Southern blot to identify cell lines carrying MMTV-*cre* transgene. Two *Hprt*(+)/MMTV-*cre*(+) cell lines from each construct were injected into C57BL/6 embryos and transferred to a B6D2 foster female. Male chimeras were mated with B6D2 females to produce mice hemizygous for the MMTV-*cre* transgene. The second construct was introduced by pronuclear injection into fertilized oocytes, generating six additional founder lines.

B. Generation of a *lox-Brca1-lox* mouse. A probe specific for exon 11 of the human *BRCA1* gene was isolated and then used to screen a mouse genomic library prepared from 129/Sv genomic DNA. A clone containing exons 11 and 12 of *Brca1* was identified and verified by sequencing. A construct containing *lox* sites flanking exon 11 of *Brca1* was then generated for targeting into ES cells. The *Hprt* gene was also inserted into this construct to facilitate positive and negative selection in future steps. Because the insertion of the *Hprt* gene into an intron of *Brca1* would likely inhibit the normal expression of BRCA1, *lox* sites were placed around the *Hprt* gene so that it could be removed after ES cell clones containing the targeted allele had been identified. This construct was electroporated into ES cells, and cells expressing the *Hprt* gene were analyzed by Southern blot to identify cell lines carrying the *lox* sites flanking exon 11 of *Brca1*. The Cre recombinase was transiently expressed in the positive clones to remove the *Hprt* locus in the targeted allele.

C. Localization of Cre expression. To establish the expression pattern of the Cre recombinase in various organs, each of the seven MMTV-*cre* mouse lines was bred to mice previously generated in our laboratory with *lox* sites surrounding exons 11 through 20 of the NMDA receptor (NR1) gene. Females which had both the MMTV-*cre* transgene as well as a *lox-Nr1-lox* allele where euthanized and DNA was isolated from the following tissues: mammary gland, salivary gland, kidney, spleen, and liver. Using PCR, the deletion of exons 11 through 20 of the *Nr1* gene, signifying the expression of cre, was determined in each of these tissues.

To localize expression within each tissue expressing the Cre recombinase, each line of *MMTV-cre* transgenic mice has been bred to a transgenic mouse which has a lacZ reporter transgene (*lacZ*). In tissues expressing the Cre recombinase, *lacZ* will be expressed in the cells will become blue when exposed to the substrate X-gal. This mouse was a generous gift from A. Berns (15). Mice containing both transgenes (*MMTV-cre* and *lacZ*) will be euthanized, and the organs of interest will be fixed and stained. These organs will then be imbedded in paraffin blocks, sectioned, and counterstained.

Technical Objective 2: Interactions of Tumor Suppressor Genes

A. Generation of *Brca1/p53*-deficient mice. The $p53^{+/-}$ mice generated by *Jacks et. al.* (9) were obtained from Jackson laboratories and were mated to F1 $Brca1^{+/-}$ animals which were derived from matings of chimeras with B6D2 females. Animals were genotyped from tail DNA using PCR protocols previously described (5,9). Mice with the following genotypes were examined for tumor formation: $Brca1^{+/+}\ p53^{-/-}$ vs. $Brca1^{+/-}\ p53^{-/-}$ mice; and $Brca1^{+/+}p53^{+/-}$ vs. $Brca1^{+/-}p53^{+/-}$ mice.

B. **γ-irradiation of BRCA1/p53-deficient mice.** *Brca1*$^{+/+}$*p53*$^{+/-}$ and *Brca1*$^{+/-}$*p53*$^{+/-}$ female mice were exposed to a single dose of 5 Gy whole body γ-irradiation between four and six weeks of age. These mice were monitored weekly for tumor formation.

C. **Tumor analysis/Histopathology.** Mice euthanized due to ill health were necropsied, and sections of tissue were snap frozen at -80° C, or fixed in 10% formalin. Tumors fixed in 10% formalin were blocked in paraffin, and slides were stained with hematoxylin and eosin and classified in a blinded fashion by a pathologist. In most cases, establishment of cell culture lines were also generated from tumor tissue sections. Non-necrotic portions of the tumors were rinsed in PBS and homogenzed in DMEM media containing 15% FBS, penicillin, streptomycin and gentamycin. The homogenized tumors were then plated on plastic 100 mm plates. Growth was monitored daily and non-adherent cells were separated from adherent cells when these two populations arose from the same tumor. Cells were passaged and growth on the above media.

D. **Loss of heterozygosity.** Non-necrotic portions of the tumors were frozen at -80° C until a number of tumors could be processed simultaneously. Tumors were then ground and homogenized in RNAzol (Tel-Test, Inc., Friendswood, Tx) and RNA was made as per the manufacturers instructions. Northern blots were generated and probed with a DNA fragment from exon 2 through 10 of *Brca1*. DNA was prepared from additional portions of the tumor using DNAzol (Life Technologies, Grand Island, NY) as per the manufacturer's instructions. DNA was digested with EcoRV and hybridized with a *Brca1* probe containing a portion of intron 9 and exon 10.

RESULTS

Technical Objective 1: Tissue-specific Inactivation of BRCA1.

A. **Generation of an MMTV-*cre* mouse.** Both MMTV-*cre* constructs were electroporated into ES cells and two positive clones from each construct was injected into C57BL/6 blastocysts. In addition, through pronuclear injection of the MMTV-*cre* transgene into fertilized oocytes, six mice carrying the MMTV-*cre* transgene were generated.

B. **Generation of a *lox-Brca1-lox* mouse**. Following the identification and cloning of exons 11 and 12 of *Brca1*, a construct was designed with *lox* sites surrounding exon 11 of *Brca1*. As described in the experimental methods section, an *Hprt* gene was also inserted into intron 10. *Lox* sites were also introduced into the construct to flank the *Hprt* gene. Following electroporation of this construct and HPRT selection, positive clones were identified. Transient expression of the Cre recombinase was accomplished by electroporation into the positive clones to generate cells which had deleted the *Hprt* gene through Cre-mediated recombination, but had left exon 11 of *Brca1* intact. Despite multiple attempts, selective Cre-mediated recombination could not be achieved. In each case, both the *Hprt* gene and exon 11 of *Brca1* were deleted.

C. **Localization of Cre expression.** MMTV-*cre* transgenic mice were bred to mice containing the targeted *Nr1* allele flanked by *lox* sites. The resulting MMTV-*cre*(+), *lox-Nr1-lox*(+) females from each MMTV-*cre* transgenic line were euthanized as virgin females or pregnant females. DNA was generated from the mammary tissue, salivary gland, spleen, liver, and kidney. PCR analysis revealed that in four of the transgenic lines, Cre was expressed in the mammary tissue of pregnant females. However, in each of the transgenic lines, Cre expression was seen in some but not all of the pregnant females. In addition, Cre expression was not seen in

the mammary tissue of virgin females in any of the lines. The four MMTV-*cre* lines which expressed the Cre recombinase in mammary tissue are currently being bred to the *lox-lacZ-lox* mice to localize expression of Cre in the mammary gland.

Technical Objective 2: Interactions of Tumor Suppressor Genes

A. Comparison of *Brca1*$^{+/+}$ and *Brca1*$^{+/-}$ mice on a p53-deficient background. *Brca1*$^{+/-}$ and *p53*$^{-/-}$ animals were intercrossed to obtain animals heterozygous for both alleles. These animals were again intercrossed and the genotype of the offspring determined. *Brca1*$^{+/+}$ and *Brca1*$^{+/-}$ mice homozygous for the mutant *p53* allele were observed at the expected frequency. As reported previously, female *p53*$^{-/-}$ mice were present in lower numbers than expected; however this was true of both *Brca1*$^{+/-}$ and *Brca1*$^{-/-}$ female pups (16,17). Mouse embryos homozygous for the mutant *Brca1* allele fail to progress past day 10 of embryo development (5). Deficiency in p53 has been reported to extend the survival of the embryos by several days (18,19). However, consistent with results reported with other *Brca1* mutant lines, p53-deficiency did not rescue the embryonic lethality of the Brca1-deficient animals. Of nine litters generated from *Brca1*$^{+/-}$*p53*$^{-/-}$ matings, no *Brca1*$^{-/-}$ offspring were observed. Genotyping of the pups identified 16 *Brca1*$^{+/+}$ and 40 *Brca1*$^{+/-}$ animals.

Thirty-eight *Brca1*$^{+/-}$ *p53*$^{-/-}$ and 40 *Brca1*$^{+/+}$*p53*$^{-/-}$ animals were monitored biweekly for the development of tumors and euthanized when behaviour and appearance indicated that death of the animals was imminent. In all cases necropsy of the moribund mice revealed obvious tumors in the dying animals. As can be seen in Figure 1, tumors arose with equal frequency and latency in both groups of animals. By 19 weeks of age, ~50% of both *Brca1*$^{+/+}$ and *Brca1*$^{+/-}$ mice on a *p53*$^{-/-}$ background had been euthanized due to tumor formation. The tumor type was determined both by observation of anatomical location, and histological examination of tumor biopsies by a trained pathologist. This analysis indicated that the distribution of tumors arising in the mice carrying a *Brca1* mutation did not differ significanly from those observed in the *Brca1*$^{+/+}$*p53*$^{-/-}$ animals. As previously reported, the predominant tumor type observed in the *p53*$^{-/-}$ mice is the thymic lymphoma (8,9). The presence of a mutant *Brca1* allele did not change the prevelance of this tumor in the *p53*$^{-/-}$ animals. Histological examination of lung, kidney, and liver from these mice also failed to reveal obvious differences between the two groups in the extent of metastases to other organ systems.

Mammary tumors were observed in both *Brca1*$^{+/+}$ and *Brca1*$^{+/-}$ mice. While the frequency of these tumors in the *Brca1*$^{+/+}$ mice was low and comparable to the frequency of such tumors seen by other investigators, four of the twenty-three *Brca1*$^{+/-}$ mice developed mammary tumors. Two of these tumors were classified as papillary adenocarcinomas, and a third was classified as a tubular mammary carcinoma. While histopathological examination established that a fourth tumor seen in this group of animals was of mammary gland origin, the characteristics of the tumor did not allow classification into established tumor subtypes. Because of the overall low number of mammary tumors, the significance of the difference in the number of tumors of this type in the two different groups of animals could not be established.

B. Analysis of *Brca1* gene expression in *Brca1*$^{+/-}$*p53*$^{-/-}$ tumors. Loss of the wild-type *BRCA1* allele is almost always observered on examination of DNA prepared from tumors arising in patients that have inherited a single mutant copy of the gene. This loss of heterozygosity thus renders the cells BRCA1-deficient, a supposed essential event during tumorigenesis (20-22). Although the similar tumor latency and spectrum in the *Brca1*$^{+/+}$ and the

Brca$^{+/-}$ mice did not suggest a role for this gene in tumorigenesis in the mouse, we wished to verify this by examination of RNA and DNA from these tumors for the expression of the wild-type *Brca1* allele. The mutation introduced into the *Brca1* gene by homologous recombination results in a smaller RNA transcript than that produced from the wild-type allele. When the wild-type *Brca1* transcript was not seen, the presence of this mutant transcript served as an internal control and was essential to determine whether loss of the heterozygosity had occurred during tumorigenesis. In tumors where analysis of RNA was not possible because of the post mortum changes in the tissue or in cases where neither the wild-type nor mutant Brca1 transcripts were seen by Northern analysis, DNA was prepared and analyzed by Southern blot for the presence of the wild-type allele. Analysis of 22 tumors from *Brca1*$^{+/-}$*p53*$^{-/-}$ mice revealed only two tumors in which loss of the wild-type allele could be detected (Table 1 and Figure 2A). As can be seen in Figure 2a, Northern analysis of mRNA prepared from the first of these two tumors failed to reveal a band corresponding to the wild-type *Brca1* transcript. In contrast, the transcipt from the mutant allele is present at high levels in the tumor RNA. Loss of homozygosity in this tumor was confirmed by DNA analysis (Figure 2B). In the second tumor Northern analysis again failed to reveal expression of the native *Brca1* transcript. While the transcript from the targeted allele was easily detected, an novel transcipt smaller in size than the wild-type *Brca1* transcript was also present in the RNA isolated from this tumor. Presumably this transcript originates from the wild-type allele which was mutated during tumorigenesis. (Figure 2C). Both of the tumors that no longer expressed wild-type *Brca1* mRNA were identified as thymic lymphomas (Table 1). None of mammary tumors examined had lost expression of the wild-type *Brca1* gene. A cell line was established from the thymic lymphoma expressing *Brca1* mRNA derived only from the targeted allele. RNA derived from this line confirmed the loss of the wild-type *Brca1* allele in this tumor (Figure 2A).

C. Tumorigenesis in *Brca1*$^{+/+}$ and *Brca1*$^{+/-}$ mice on a *p53*-heterozygous background. We considered the possibility that the high incidence and early appearance of thymic lymphomas leading to the early death of the majority of the *Brca1*$^{+/-}$*p53*$^{-/-}$ and wild-type mice may obscure our ability to observe cooperativity between these two tumor suppressor genes in mammary tumorigenesis. While *p53*$^{+/-}$ mice are predisposed to tumors, the tumor latency and lifespan of *p53*$^{+/-}$ mice is longer. We therefore also examined tumor formation in *Brca1*$^{+/+}$ and *Brca1*$^{+/-}$ littermates on a *p53*$^{+/-}$ background. Twenty-three mice heterozygous for both the *p53* and *Brca1* mutation were generated, and survival and tumor formation were compared to twenty-three mice carrying only a single copy of the mutant *p53* gene. Similar to our observations of the *Brca1*$^{+/-}$ mice on a *p53*$^{-/-}$ background, germline loss of one copy of *Brca1* does not alter the survival or the types of tumors that arise in the *p53*$^{+/-}$ animals (Table 2 and Figure 3).

RNA was isolated from 6 tumors that arose in the *Brca1*$^{+/-}$ *p53*$^{+/-}$ mice. In all cases, Northern analysis revealed the presence of both the wild-type *Brca1* transcript and the transcript originating from the targeted allele.

D. Tumorigenesis in irradiated BRCA1- and p53- deficient mice. As it is possible that exposure to specific environmental agents together with the inheritance of mutations in tumor suppressor genes are essential for tumor formation in humans, we studied the effect of gamma irradiation on the tumor latency and tumor distribution in the BRCA1/p53 deficient animals. Our observations, consistent with published work, showed that irradiation of *p53*$^{-/-}$ mice decreases the age at which the thymic lymphomas appear (23). The accelerated formation of thymic lymphomas would likely hinder our ability to observe the role of p53 and

BRCA1 in mammary tumor formation. Therefore, this study was carried out with BRCA1-deficient mice heterozygous for the *p53* mutation. Seventeen female *Brca1*$^{+/+}$ and twenty-one female *Brca1*$^{+/-}$ littermates heterozygous for the *p53* mutation were exposed to a single dose of 5 Gy whole body γ irradiation between four and six weeks of age. At this age the murine mammary epithelium is proliferating and is believed to be more sensitive to DNA damaging agents. Mice were observed bi-weekly, killed when moribund, and autopsied. Compared to the unirradiated *p53*$^{+/-}$ mice, the age of onset of tumor formation was decreased in both *Brca1*$^{+/+}$*p53*$^{+/-}$ and *Brca1*$^{+/-}$*p53*$^{+/-}$ mice (compare Figures 3 and 4). However, as observed with the unirradiated populations, loss of one *Brca1* allele did not affect the survival rate of the *p53* heterozygotes (Figure 4). Consistent with published reports the tumor spectum of the irradiated *p53*$^{+/-}$ animals differs from the untreated animals in that the predominant tumor seen is the thymic lymphoma (23). This finding was observed in both the irradiated *Brca1*$^{+/+}$*p53*$^{+/-}$ and *Brca1*$^{+/-}$*p53*$^{+/-}$ populations of mice (Table 3). While none of the irradiated *Brca1*$^{+/+}$*p53*$^{+/-}$ mice developed mammary tumors, five mammary tumors, verified by histopathology, were observed in irradiated *Brca1*$^{+/-}$*p53*$^{+/-}$ mice.

E. Brca1 expression in mammary tumors arising in irradiated *Brca1*$^{+/-}$*p53*$^{-/-}$ animals. Five tumors arising in the irradiated *Brca1*$^{+/-}$ *p53*$^{+/-}$ population, including 2 of the mammary tumors, were examined for loss of heterozygosity at both the *p53* and *Brca1* locus. To examine loss of heterozygosity of p53, DNA prepared from each sample was analysed by Southern blot using a probe specific for exons 7 through 9 of the *p53* locus. In all five tumors the genomic fragment corresponding to the wild-type *p53* allele was absent.

Loss of heterozygosity at the *Brca1* locus was examined as described above using RNA isolated from tumors. Northern analysis of RNA prepared from the three ovarian tumors, a single histiocytoma, a pilomatricoma and a hemangiosarcoma showed that expression of the *Brca1* wild-type allele was unaltered in these tumors. Similarly RNA analysis of two of the mammary carcinomas showed expression of both the mutant and the wild-type *Brca1* genes in the tumor tissue (data not shown). Analysis of a third mammary tumor yielded different results. RNA isolated from this tumor, classified as a papillary adenocarcinoma, indicated that the wild-type *Brca1* allele was no longer expressed, while transcripts originating from the targeted allele were easily visable. Loss of heterozygosity was demonstrated by analysis of DNA obtained from the tumor, as only the band corresponding to the mutant allele was observed on Southern analysis. RNA analysis revealed that the loss of the wild-type allele of *Brca1* had occurred in the remaining two mammary tumors. Histological analysis revealed one tumor was a mammary intraductal carcinoma and the second was an anaplastic tumor of unknown origin. The high levels of cytokeratin 18 mRNA in the anaplastic tumor, taken together with the subcutaneous location in the mammary gland region, suggests that this tumor is of mammary origin. The loss of wild-type BRCA1 expresson in three of the five mammary tumors in the irradiated *Brca1*$^{+/-}$ *p53*$^{+/-}$ mice and the lack of mammary tumors in the *Brca1*$^{+/+}$*p53*$^{+/-}$ mice suggests a correlation between *Brca1* status and the formation of mammary tumors. Because of the small number of mammary tumors obtained in these studies differences between the number of tumors in the two groups does not achieve statistical significance.

DISCUSSION

Technical Objective 1: Tissue-specific Inactivation of BRCA1. The establishment of MMTV-*cre* transgenic lines which express the Cre recombinase in mammary epithelial tissue will be useful not only in understanding the role of BRCA1 in mammary tumorigenesis, but the role of other tumor suppressors as well, such as BRCA2 and p53. Though the generation of the *lox-Brca1-lox* mouse has been problematic, using the *lox-Nr1-lox* mouse and the *lox-lacZ-lox* mouse, we will be able to identify the MMTV-*cre* transgenic mouse which has the highest and most uniform expression in mammary epithelium. We will also be able to determine in which cell types cre is being expressed. This will facilitate the generation of the most informative MMTV-*cre*(+)/*lox-Brca1-lox*(+) mice.

Although we have successfully generated ES cells in which *lox* sites flank an essential exon of *Brca1*, the inability to remove the *Hprt* gene from the targeted allele will most likely result in an inability to express BRCA1 properly from the genome. Thus, even in cells not expressing the Cre recombinase, homozygosity of the targeted allele will likely lead to BRCA1 deficiency. In addition to refining the technique to allow transient Cre expression in the positive cells, two additional constructs will be designed. The first construct will target the 3' end of the *Brca1* gene. Because germline mutations in *Brca1* seen in ovarian cancer patients are frequently in 3' end of the *BRCA1* gene and because the carboxy terminal end of BRCA1 is highly conserved with the mouse homologue, the terminal exons are likely to be crucial in the function of BRCA1. In this construct, the *Hprt* gene will be placed following the polyadenylation signal and will therefore not disturb the expression of BRCA1. *Lox* sites will therefore only surround the exons of *Brca1* and selective cre-mediated recombination will not be necessary. The second construct will contain the *Brca1* cDNA surrounded by *lox* sites. Using reverse transcriptase followed by PCR, we have generated a *Brca1* cDNA and have isolated the region upstream of the BRCA1 gene. A single copy of the transgene will be targeted into the *Brca1* locus. When the cDNA portion is flanked by *lox* sites, tissue specific removal of the transgene will be possible.

Technical Objective 2: Interactions of Tumor Suppressor Genes. To determine whether mutations in *Brca1* would alter the frequency and type of tumors formed in the p53-deficient mice, we have generated mice carrying mutations at both of these loci. In addition we examined the effect of ionizing radiation on tumor formation in these animals. We found that survival of both $p53^{-/-}$ and $p53^{+/-}$ mice is not altered by the presence of a mutant *Brca1* allele. Only two tumors were found which showed loss of the wild-type allele and the characteristics including the latency of these tumors did not differ from those in which wild-type BRCA1 expression was seen. This suggests that deficiency in BRCA1 does not play a role in the formation of most tumors. This is perhaps not surprising when one considers that inheritance of a mutant *BRCA1* allele in humans leads to increased risk for very specific tumor types, primarily ovarian and breast tumors. Although no major changes in the tumor types were noted in either the $Brca1^{+/-}p53^{-/-}$ or $Brca1^{+/-}p53^{+/-}$ populations, four mammary tumors were seen in the $Brca1^{+/-}p53^{-/-}$ group while only one such tumor was seen among the control $p53^{-/-}$ group.

It has been suggested that BRCA1 is involved in DNA repair particularly after exposure to gamma irradiation. We found that the survival rate of the $p53^{+/-}$ mice after exposure to gamma irradiation was not altered by the presence of a mutant *Brca1* allele. However while none of the 14 irradiated $p53^{+/-}$ mice developed mammary tumors, five of the 23 tumors isolated from the BRCA1-deficient mice were of mammary epithelial origin, and of these three had lost expression

of the wild type *Brca1* gene. Although the number of mammary tumors obtained is small, these results are suggestive of a role for BRCA1 in mammary tumor formation after exposure to specific DNA damaging agents.

RECOMMENDATIONS REGARDING THE STATEMENT OF WORK

Technical Objective 1: Tissue-specific Inactivation of Brca1. The difficulty in generating a *lox-Brca1-lox* mouse has hindered our attempts to complete this objective within the time frame originally proposed. However using the *lox-Nr1-lox* mouse and the *lox-lacZ-lox* mouse, we will be able to identify the *MMTV-cre*(+) mouse line which expresses the cre recombinase in mammary glands, but not in other tissues. We will also be able to clearly distinguish which cell types and the what percentage of these epithelial cells are expressing the cre protein. We are also redesigning the *lox-Brca1-lox* construct in order to generate a *lox-Brca1-lox* mouse.

Technical Objective 2: Interactions of Tumor Suppressor Genes. By the first annual review of this grant, we have completed all of the tasks proposed under this technical objective. The generation of tumor lines, specifically two of mammary epithelial origin, will now allow us to more clearly define the importance of BRCA1 in normal cell growth and tumorigenesis. Although many *BRCA1*$^{-/-}$ mammary tumors have been discovered in humans, these cells have thus far been reticent to cell line formation. To date, only one *Brca1*$^{-/-}$ cell line, an ES cell line, has been reported (5). The generation of BRCA1-deficient cell lines will enhance our ability to understand the functions of BRCA1 and what pathways are eliminated in BRCA1-deficient tumors.

These cell lines will be used to test two prevalent hypotheses regarding BRCA1 function in cell cycle regulation and direct DNA repair by analysis of the normal growth and response to DNA damaging elements. Initial studies will focus on the role of BRCA1 in malignant cell growth. Previous studies have shown that when expressed in a constitutive manner, BRCA1 can cause cell death in cell lines derived from tumors (24). To determine if the murine homologue BRCA1 plays a similar role, *Brca1*$^{+/-}$ and *Brca1*$^{-/-}$ mammary tumor cell lines will be transfected with a *Brca1*-cDNA construct driven by a CMV promoter. If cell death results from the constitutive production of BRCA1, a second construct containing the *Brca1* cDNA driven by its native promoter will be independently transfected into the *Brca1*$^{+/-}$ and *Brca1*$^{-/-}$ mammary cell lines to determine if cellular death is resulting from the inappropriate production of BRCA1 throughout the cell cycle. *Brca1*$^{-/-}$ cells which have stably integrated the *Brca1* construct will be isolated and expanded to provide a good control cell line for the *Brca1*$^{-/-}$ cell line. Studies focusing on the growth and senescense of the *Brca1*$^{-/-}$*p53*$^{-/-}$ mammary cell lines can then be pursued. A complication of these studies is the fact that these cells are deficient in p53, which itself will have a major impact on cell growth and response to DNA damage. Nevertheless, with the inclusion of appropriate controls, important information will be obtained from the analysis of these cells.

CONCLUSIONS

Technical Objective 1: Tissue-specific Inactivation of BRCA1. Using two MMTV-*cre* constructs, we have identified four different mouse lines which express the Cre recombinase in the mammary glands. We are currently using a mouse with a reporter transgene, *lacZ*, to identify the mammary epithelial cell types in which the Cre recombinase is expressed. Although we have

been unable to generate *lox-Brca1-lox* mice, we are using three different approaches to accomplish this aim.

Technical Objective 2: Interactions of Tumor Suppressor Genes. The generation of mice deficient in both BRCA1 and p53 has allowed us to examine possible synergistic effects of these two tumor suppressors in tumorigenesis. The similarity in the tumor spectrum and rate of tumor formation in mice with a *Brca1* mutant allele suggests that mutations in both BRCA1 and p53 are not sufficient for mammary tumorigenesis to occur. However, loss of the wild-type allele of BRCA1 in three of the five mammary tumors in irradiated $Brca1^{+/-}p53^{+/-}$ female mice is suggestive of a role for BRCA1 in murine mammary tumorigenesis. Because the low frequency of mammary tumors precluded statistical analysis in this study, we are continuing this study with additional $Brca1^{+/-}p53^{+/-}$ mice in the hopes of generating enough mammary tumors to define a role for BRCA1 in murine mammary tumorigenesis.

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Appendix A: FIGURE LEGENDS

Figure1. Germline loss of *Brca1* does not affect the survival rate of $p53^{-/-}$ mice. $Brca1^{+/+}$ and $Brca1^{+/-}$ mice on a $p53^{-/-}$ background were carefully monitored for ill health. Moribund mice or mice with large tumors were euthanized and necropsy was performed. $Brca1^{+/+}p53^{-/-}$ mice: black line, n=40; $Brca1^{+/-}p53^{-/-}$ mice: gray line, n=38.

Figure 2. Loss of Brca1 heterozygosity in p53-deficient tumors. RNA was collected from tumor samples and cell lines derived from tumors run on a Northern blot, and probed with exons 2 through 11 of *Brca1*. The lower band ("mutant") is the RNA derived from the germline mutated allele. Its presence acts as an internal control. (A) RNA from one thymic lymphoma reveals the deletion of the wild-type copy of *Brca1*. RNA from the cell line derived from this tumor revealed that it was also BRCA1-deficient. (B) DNA derived from this tumor confirms the loss of the *Brca1* allele. (C) Northern analysis reveals that another thymic lymphoma line deleted a portion of *Brca1*. (D) RNA made from one mammary tumors of irradiated $Brca1^{+/-}p53^{+/-}$ mice revealed the loss of the wild type *Brca1* allele. (E) Southern analysis confirmed the loss of heterozygosity of *Brca1*.

Figure 3. Germline loss of *Brca1* does not affect the survival rate of $p53^{+/-}$ mice. $Brca1^{+/+}$ and $Brca1^{+/-}$ mice on a $p53^{+/-}$ background were carefully monitored for ill health. Moribund mice or mice with large tumors were euthanized and necropsy was performed. $Brca1^{+/+}p53^{+/-}$ mice: black line, n=23; $Brca1^{+/-}p53^{+/-}$ mice: gray line, n=23.

Figure 4. Gamma irradiation of mice alters the overall tumor latency of $p53^{+/-}$ mice but affects $Brca1^{+/+}$ and $Brca1^{+/-}$ mice similarly. Female $Brca1^{+/+}$ and $Brca1^{+/-}$ mice on a $p53^{+/-}$ background were irradiated with 500 rads between 4-6 weeks of age. Moribund mice or mice with obvious tumors were euthanized and mice were necropsied. Although overall survival was decreased in both groups of p53+/- mice, the viability of mice was similar in the $Brca1^{+/+}$ and $Brca1^{+/-}$ mice. $Brca1^{+/+}p53^{+/-}$, n=17; $Brca1^{+/-}p53^{+/-}$, n=21.

Appendix B: Figures

Figure 1

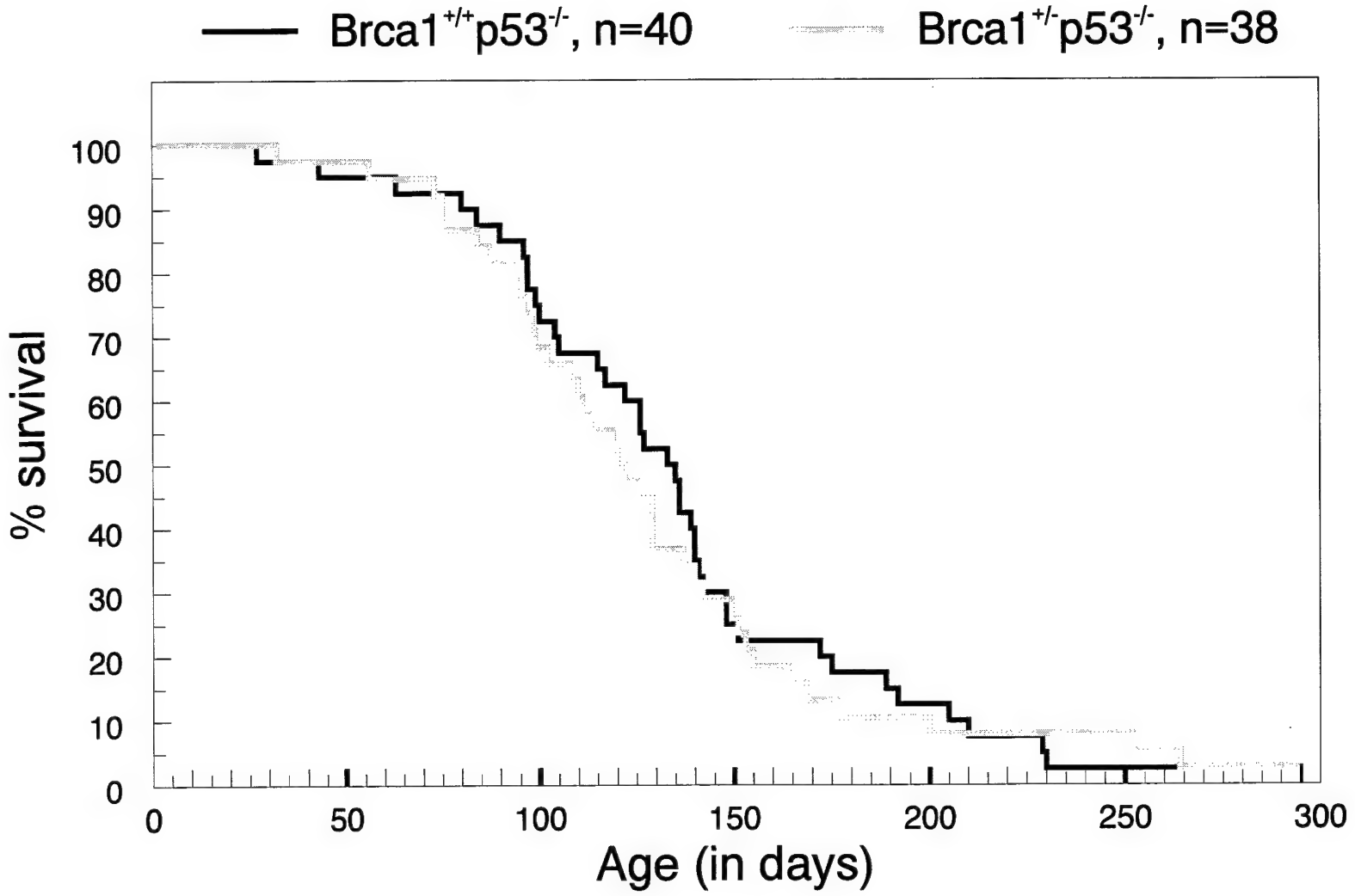
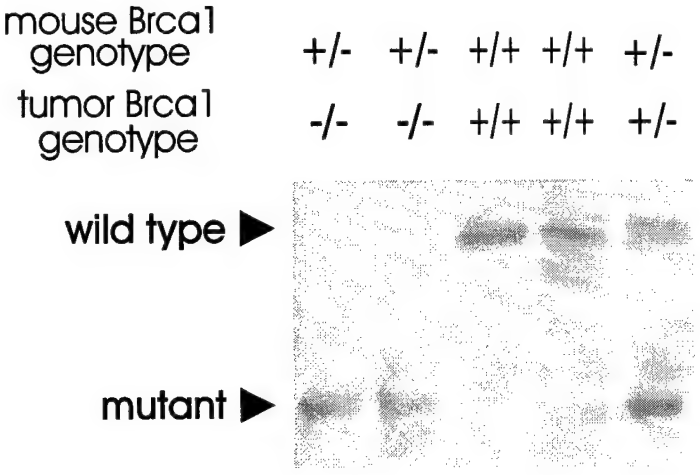

Brca1+/+p53-/-, n=40
Brca1+/-p53-/-, n=38
% survival
100
90
80
70
60
50
40
30
20
10
0
0
50
100
150
200
250
300
Age (in days)

Figure 2

A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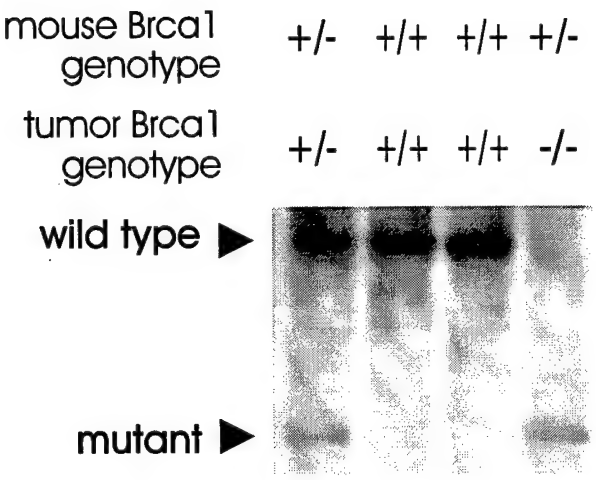
+/- +/- +/+ +/+ +/-
tumor Brca1 genotype
-/- -/- +/+ +/+ +/-
wild type
mutant

B


mouse Brca1 genotype
+/- +/+ +/+ +/-
tumor Brca1 genotype
+/- +/+ +/+ -/-
wild type
mutant

C

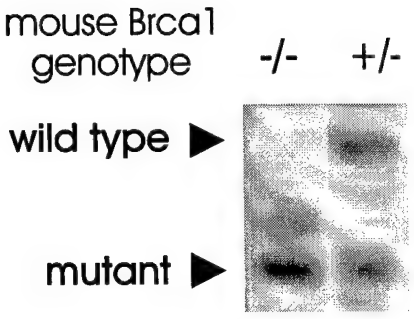
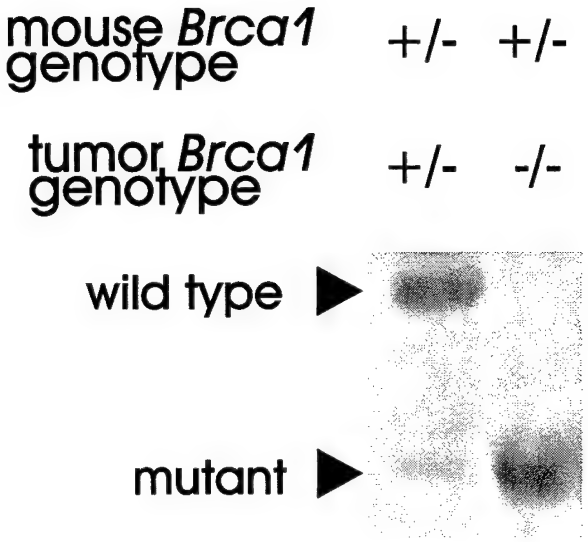

mouse Brca1 genotype
-/- +/-
wild type
mutant

Figure 2 (cont.)

D.



E.

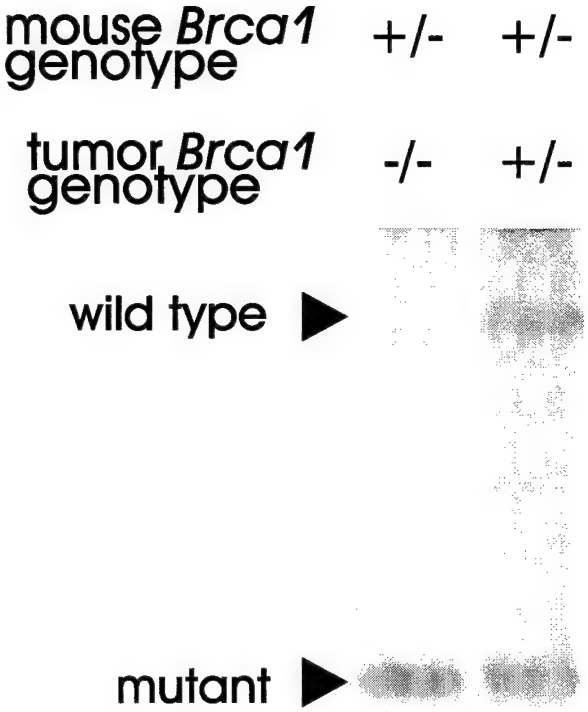


Figure 3

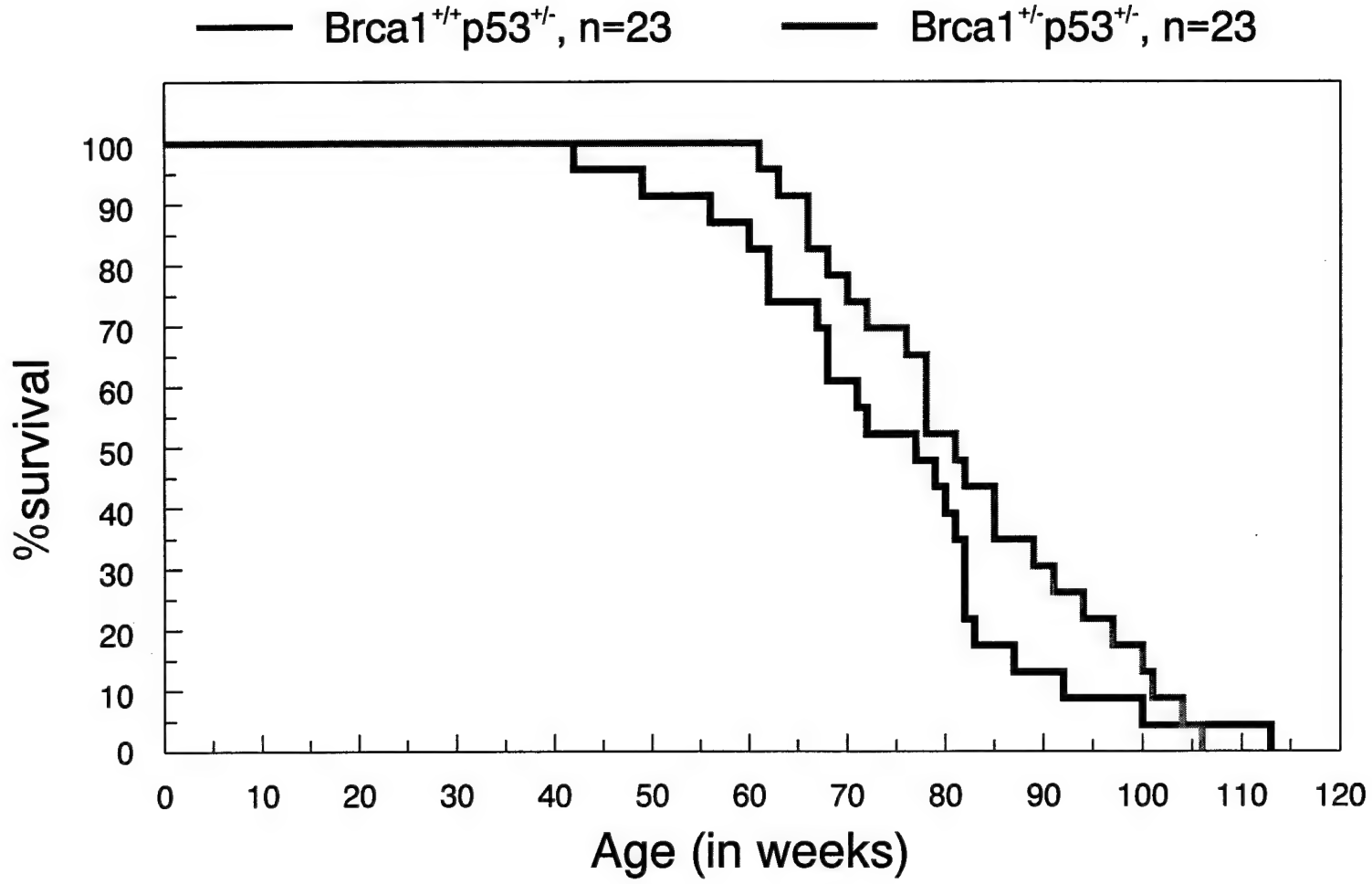
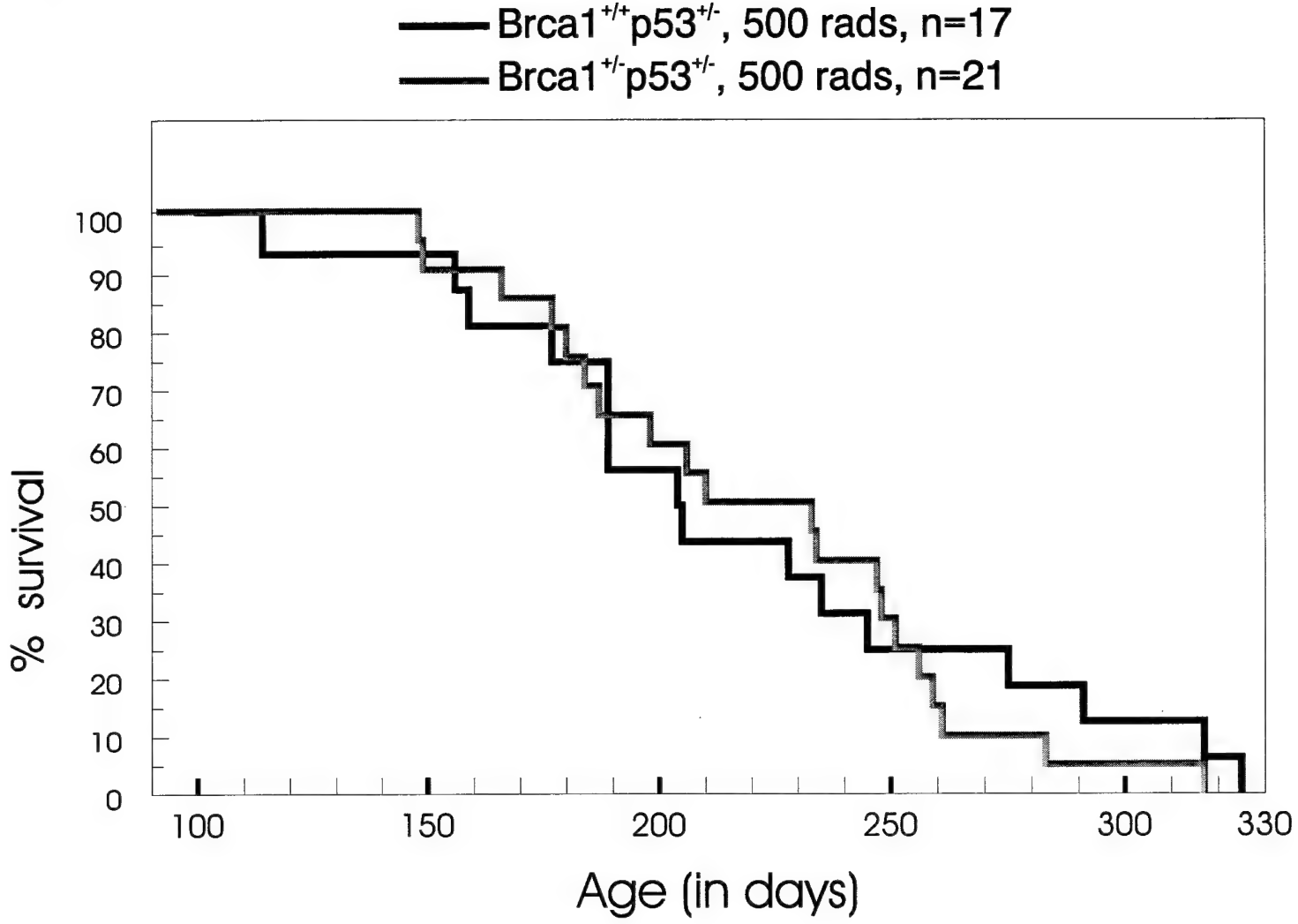

Brca1+/+p53+/-, n=23
Brca1+/-p53+/-, n=23
%survival
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
110
120
Age (in weeks)

Figure 4


Brca1+/+p53+/-, 500 rads, n=17
Brca1+/-p53+/-, 500 rads, n=21
% survival
100
90
80
70
60
50
40
30
20
10
0
100
150
200
250
300
330
Age (in days)

Apppendix C: Tables

Table 1: Tumor spectrum in Brca1+/+ vs. Brca1+/- mice on a p53-/- background

	Brca1+/+		Brca1+/-		# Brca1-/- tumors / # tumors examined for LOH
	n=51	percentage	n=41	percentage	
thymic lymphoma	28	54.9	19	46.3	2 / 9
lymphoma	9	17.6	10	24.4	0 / 6
osteosarcoma	1	2.0	-	-	-
hemangiosarcoma	3	5.9	-	-	-
cerebellar defect	2	3.9	2	4.9	-
adenocarcinoma of the Hardarian gland	1	2.0	-	-	-
rhabdomyosarcoma	1	2.0	-	-	-
teratoma	2	3.9	2	4.9	0 / 2
mammary carcinoma	1	2.0	4	9.8	0 / 3
adenocarcinoma of rete ovarii	-	-	1	2.4	0 / 1
carcinoma of unknown origin	1	2.0	2	4.9	0/ 6
no tumors	2	3.9	1	2.4	-

Table 2: Tumor spectrum in Brca1+/+ vs. Brca1+/- mice on a p53+/- bactground

	Brca1+/+		Brca1+/-		# Brca1-/- tumors
	n=19	percentage	n=23	percentage	# tumors examined for LOH
osteosarcoma	7	36.8	5	21.7	-
leiomyosarcoma	2	10.5	-	-	-
reticulum cell sarcoma	2	10.5	1	4.3	0 / 1
bronchoalveolar carcinoma	2	10.5	2	8.7	-
hepatocellular carcinoma	1	5.3	2	8.7	-
mammary carcinoma	1	5.3	-	-	-
keratoacanthoma	1	5.3	-	-	-
basal cell carcinoma	-	-	2	8.7	0 / 2
fibrosarcoma	-	-	4	17.4	0 / 2
hemangiosarcoma	-	-	2	8.7	-
squam cell carcinoma	-	-	1	4.3	-
spindle cell tumor	-	-	1	4.3	-
non-tumor related death	3	15.8	3	13.0	-

Table 3: Tumor spectrum in irradiated Brca1+/+ vs. Brca1+/- mice on a p53+/- background

	Brca1 +/+		Brca1 +/-		# Brca1-/- tumors / # tumors examined for LOH
	n=14	percentage	n=24	percentage	
thymic lymphoma	8	53.3	10	41.7	-
lymphoma	1	6.7	-	-	-
brain tumor	1	6.7	-	-	-
ovarian	2	14.3	3	12.5	0 / 3
sarcoma	1	6.7	2	8.3	-
mammary	-	-	5	20.8	3 / 5
fibrous histiocytoma	-	-	1	4.2	0 / 1
pilomatricoma	-	-	1	4.2	0 / 1
hemangiosarcoma	-	-	1	4.2	0 / 1
non tumor	1	6.7	1	4.2	-